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# IMMUNOLOGY

## FOURTH EDITION

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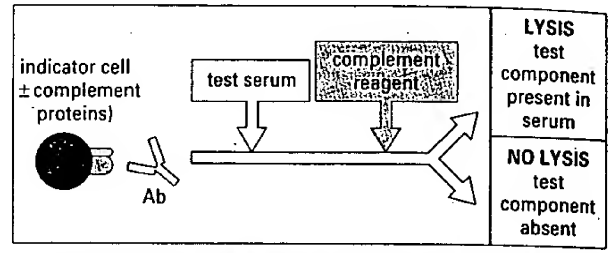
Although a monoclonal antibody is a well-defined reagent it does not have a greater specificity than a polyclonal antiserum which recognizes the antigen by means of a number of different epitopes.

## ■ ASSAYS FOR COMPLEMENT

The simplest measurement of complement activity is to determine the concentration of serum which will cause lysis of 50% of a standardized preparation of antibody-sensitized erythrocytes (EA). This is carried out in tubes or microwells. A simpler system, which provides a crude measure of complement activity is single-radial haemolysis. The technique is similar to that of single-radial immunodiffusion (see *Fig. 28.4*) except that the wells contain the test serum and the gel contains EA. A zone of haemolysis develops around wells containing active complement, and the size of the zone is proportional to the amount of complement in the well. This technique measures the total activity of the classical and lytic pathways (C1–C9), but if a serum is deficient in complement activity it cannot identify which complement protein is lacking.

Individual components may be measured separately to determine either their total level or their functional level. This is an important distinction, since a component may be present in normal quantities but be functionally inactive. Total levels of individual complement proteins are usually measured by RIA or by ELISA using antibody specific for the protein under investigation. Functional levels are measured in assays tailored to detect each individual complement protein by providing a cocktail of sensitized red cells plus all the components required for lysis, except the one under investigation (*Fig. 28.18*).

**Assays for complement components**




| test | indicator | complement reagent |
|---|---|---|
| C1 | EAC 4 (guinea-pig) | C1 reagent |
| C4 | EA | C4-deficient guinea-pig serum |
| C2 | EAC 4 (human) (antrypol) | C2 reagent |
| C3 | EAC 142 (guinea-pig) | C5–9($NH_3$ treated guinea-pig serum) |
| C5 | EAC 14 oxy 23 | C5-deficient mouse serum |
| C6 | EAC 143 (human) (antrypol) | C6-deficient rabbit serum |
| FB | EA + EGTA + $Mg^{2+}$ | B-deficient serum (50°C treated) |
| FD | EA + EGTA + $Mg^{2+}$ | D-deficient serum (Sephadex G75 exclusion peak) |

**Fig. 28.18.** These assays detect specific complement components in a test serum. The principle of the assay is to mix sensitized red cells with a 'complement reagent' so that the sensitized cells plus the reagent contain all the complement components needed to lyse the red cells except for the component being tested. For example, to test for C4, erythrocytes sensitized with antibody (EA) are placed with C4-deficient guinea-pig serum. The cells will be lysed if there is C4 in the test serum, but not if none is present. The table lists the combinations of reagents used for each test component. The red cells are prepared by blocking the reactions of EA with complement at a specific point. The complement reagents may be sera thought to be deficient in one component or sera treated physicochemically to remove or inactivate one component. In practice the assay would be performed quantitatively, for example, by single-radial haemolysis, or in tubes to determine the point at which 50% of the red cells are lysed.

## ■ ISOLATION OF LYMPHOCYTE POPULATIONS

Many of the experiments performed by immunologists use populations of lymphocytes for work either *in vivo* or *in vitro*. The main sources of lymphocytes from experimental animals are the thymus, the spleen or the peripheral lymph nodes. Specialized studies may require isolation of cells from other areas such as Peyer's patches. Recirculating cells may be obtained by cannulating the thoracic duct and collecting the draining lymphocytes over a number of hours. In studies on humans, peripheral blood lymphocytes are the most readily available source of cells, but spleen, tonsil or lymph nodes may become available following surgical resection. However, problems can arise with surgical material due to the presence of infectious agents or tumour cells, depending on the circumstances that led to surgery. It should be emphasized that the cell populations derived from each of these tissues is quite distinct, with respect to the maturity of the lymphocytes and the proportions of different cell populations. The thymus is a source of fairly pure T cells but these are at varying stages of maturity. When working on lymphocytes from other sources, it is often desirable to separate the different cell populations so as to distinguish their effects.

Reference has already been made to the use of the fluorescence-activated cell sorter (FACS) for the isolation of lymphocyte populations, based on their surface markers. The number of cells isolated is, however, limited by the flow-through rate, which is slow because each cell is individually sorted. A number of bulk methods are also available for separating lymphocytes and the specific subpopulations. These include density-gradient separation, rosetting, panning and magnetic separations.

Density-gradient separation relies on lymphocytes being less dense than erythrocytes and granulocytes (*Fig. 28.19*), and is used to isolate the majority of blood lymphocytes.

Rosetting and panning (or plating) are used to isolate subpopulations (*Figs 28.20* and *28.21*). Lymphocyte panning is a type of affinity chromatography applied to lymphocytes. A related technique uses magnetic beads coated with specific antibodies (e.g. anti-CD4). The beads are mixed with the cell population and bind to those recognized by the antibody. These cells can then be removed or isolated by applying a magnetic field.

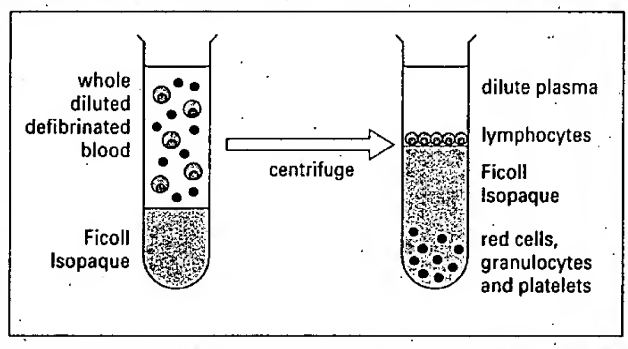
Another useful method for removing unwanted cell populations relies on antibody and complement. When a specific antibody (e.g. anti-CD8) is added to a mixture of cells, followed by complement, that subpopulation of cells will be lysed. Naturally this will only work with antibodies that fix complement, and where the target population of cells has sufficient surface antigens to fix a lytic dose of complement.

Another approach to the preparation of lymphocytes is to generate antigen-specific lines of T cells, and propagate them for an extended period (*Fig. 28.22*). This obviates the need for frequent isolation of primary cultures from animals.

## ■ EFFECTOR-CELL ASSAYS

Various methods have been developed for assaying lymphocyte-effector functions, including antibody production, cytotoxicity, and T-cell-mediated help and suppression.

**Density-gradient separation of lymphocytes on Ficoll Isopaque**



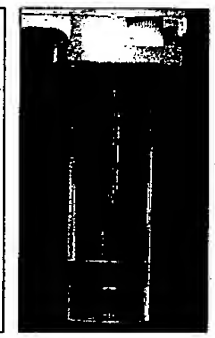
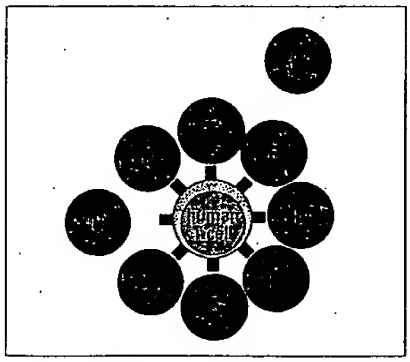



**Fig. 28.19** Lymphocytes can be separated from whole blood using a density gradient. Whole blood is defibrinated by shaking with glass beads and the resulting clot removed. The blood is then diluted in tissue culture medium and layered on top of a tube half full of Ficoll. Ficoll has a density greater than that of lymphocytes but less than that of red cells and granulocytes (e.g. neutrophils). After centrifugation the red cells and polymorphonuclear neutrophils (PMNs) pass down through the Ficoll to form a pellet at the bottom of the tube while lymphocytes settle at the interface of the medium and Ficoll. The lymphocyte preparation can be further depleted of macrophages and residual PMNs by the addition of iron filings; these are taken up by phagocytes which can then be drawn away with a strong magnet. Macrophages can be removed by leaving the cell suspension to settle on a plastic dish. Macrophages adhere to plastic, whereas the lymphocytes can be washed off.

**Isolation of lymphocyte subpopulations – rosetting**



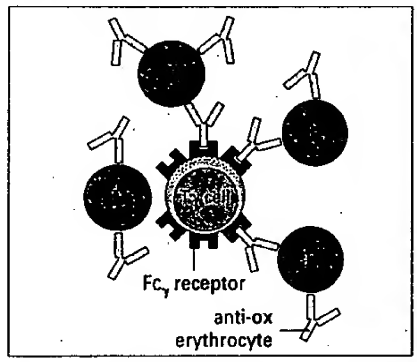




**Fig. 28.20** Rosetting relies on the fact that some lymphocyte populations have receptors for erythrocytes. Human T cells have receptors for sheep erythrocytes (E); these are CD2 molecules (left). They are not present on mouse T cells in sufficient quantities, so mouse T cells cannot be isolated by this approach. When mixed together the T cells form rosettes with the erythrocytes and may be separated from non-rosetting B cells on Ficoll gradients. A modification of this technique to isolate cells with other receptors is also shown (middle). For example, some T cells (Tγ cells) have a receptor for the Fc of IgG ($Fc\gamma$). These cells may be identified and isolated by rosetting with ox erythrocytes sensitized with a subagglutinating amount of anti-ox erythrocyte. A rosetted lymphocyte is shown on the right. (Courtesy of Dr P. M. Lydyard.)

**Isolation of lymphocyte subpopulations – panning**

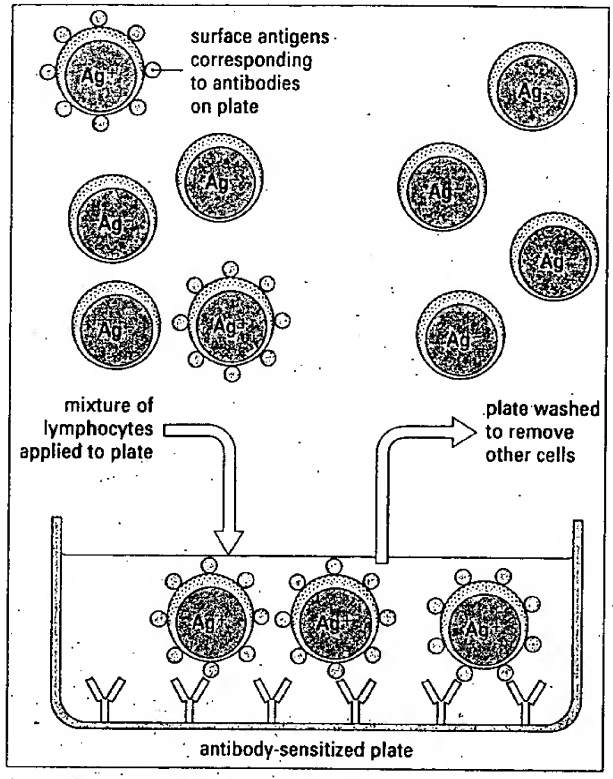



**Fig. 28.21** Cell populations can be separated on antibody-sensitized plates. Antibody binds non-covalently to the plastic plate (as for solid-phase immunoassay) and the cell mixture is applied to the plate. Antigen-positive cells ($Ag^+$) bind to the antibody and the antigen-negative cells ($Ag^-$) can be carefully washed off. By changing the culture conditions or by enzyme-digestion of the cells on the plate it is sometimes possible to recover the cells bound to the plate. Often the cells that have bound to the plate are altered by their binding; for example, binding to the plate cross-links the antigen which can cause cell activation. Thus, the method is most satisfactory for removing a subpopulation from the population, rather than isolating it. Examples of the application of this method include separating $T_H$ and $T_C$ cell populations using antibodies to CD4 or CD8, and separating T cells from B cells using anti-Ig (which binds to the surface antibody of the B cell). In reverse, by sensitizing the plate with antigen, antigen-binding cells can be separated from non-binding cells.

**T-cell lines**

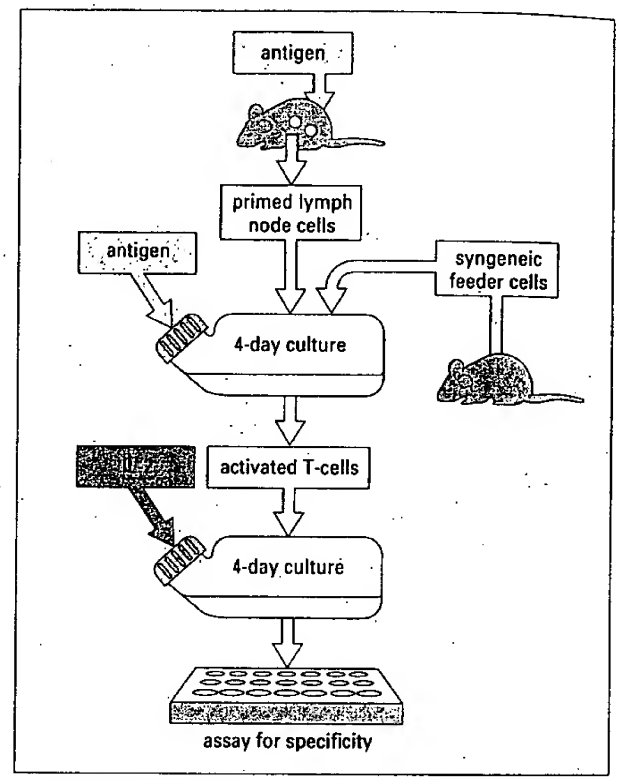



**Fig. 28.22** The figure illustrates one protocol for the preparation of T-cell lines, although many other protocols are used. Mice are primed with antigen (usually subcutaneously in the rear foot pad), and the draining lymph nodes (in this case the popliteal and inguinal) are removed 1 week later and set up in co-culture with the antigen and with syngeneic feeder cells, i.e. cells from mice of the same inbred line (e.g. normal thymocytes or splenocytes). After 4 days the lymphoblasts are isolated and induced to proliferate with interleukin-2 (IL-2). When the population of cells has expanded sufficiently they are checked for antigen and MHC specificity in a lymphocyte transformation test, and are maintained by alternate cycles of culture on antigen-treated feeder cells and culture in IL-2-containing medium.

Antibody-forming cells are measured by plaque-forming cell assay (*Fig. 28.23*), which can detect IgG- or IgM-producing cells. Another way of detecting antibody-producing cells is by the ELISPOT enzymatic test assay (*Fig. 28.24*). A development of this assay allows the detection of functional T cells according to the soluble mediators they release, i.e. cytokines. In this assay the plate is sensitized with an antibody to the specific cytokine (e.g. anti-IFN). This captures the specific cytokine released in a spot around the active T cell.

Antigen-specific T cells are often detected by the lymphocyte stimulation test, which measures their response to antigen as shown by their entering the cell cycle and incorporating precursors of DNA synthesis (*Fig. 28.25*). The cytotoxic activity of cell populations is usually detected by their ability to lyse target cells (e.g. virally infected cells, tumour cells, allogeneic tissue cells). Target-cell lysis is determined in the chromium-release assay (*Fig. 28.26*).

## Lymphocyte migration

Experiments for the detection of lymphocyte migration *in vivo* usually involve tracking of labelled lymphocytes to particular tissues after intravenous infusion. The cells may be radiolabelled or marked with stable fluorescent dyes.